CHEMICAL CHARACTERIZATION OF EUCALYPT PULP LIGNIN DURING TOTALLY CHLORINE FREE BLEACHING INCLUDING A LACCASE-MEDIATOR STAGE: 2D-NMR, FTIR AND Py-GC/MS OF ENZYMATICALLY-ISOLATED RESIDUAL LIGNINS

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ABSTRACT

Chemical modification of residual lignin isolated from eucalypt pulp after the different stages of a TCF sequence including a laccase-mediator stage was investigated by FTIR, Py-GC/MS and 2D-NMR (using HSQC, HSQC-TOCSY and HMBC). The enzymatic treatment resulted in strong oxidative modification of a lignin fraction, which could be removed under alkaline conditions enabling a final increase of brightness by hydrogen peroxide. In addition to changes in the ratios between syringyl and guaiacyl units and between β-O-4, syringaresinol and phenylcoumaran substructures, formation of acetosyringone and muconate terminal units were observed during the TCF sequence due to oxidative breakdown of lignin side-chains and aromatic nuclei respectively.

I. INTRODUCTION

World consumption of eucalypt wood for paper pulp has strongly increased during last years, including production of high-quality TCF pulps. However, the delignification and brightness levels attained after TCF bleaching of eucalypt kraft pulp are lower than obtained with ECF bleaching. Oxidative enzymes have high potential for delignifying paper pulps, especially the so-called laccase-mediator system, and can be combined with chemical reagents in new TCF sequences. For optimizing the enzymatic delignification of pulp it is important to know the modifications produced in lignin chemical structure. Acidolysis has been used for isolating lignin from paper pulps but enzymatic isolation represents an attractive alternative due to its milder nature (Ibarra et al., 2004). In this study residual lignins were enzymatically-isolated from eucalypt (*Eucalyptus globulus*) pulp during a laboratory TCF sequence (Ibarra et al., 2006) that included an enzymatic stage using laccase and HBT, and analyzed by FTIR, Py-GC/MS and 2D-NMR.

II. EXPERIMENTAL

The enzyme-containing bleaching sequence was assayed on 200 g of eucalypt kraft pulp, with a kappa number of 14.2 and a brightness of 41.2% ISO, in 4-L pressurized reactors at 10% consistency. The enzymatic stage (L) consisted of 2 h at pH 4 and 50ºC (1-min stirring at 60 rpm every 30 min) with 20 U/g (pulp dry weight) of laccase from *Pycnoporus cinnabarinus* (activity measured on ABTS at pH 5) and 1.5% HBT (pulp dry weight). The TCF sequence (O-O-L-Q-PoP) included two oxygen stages, a chelation stage, and a double alkaline peroxide stage (the first step under oxygen) (Ibarra et al., 2006). The control sequence (O-O-a-Q-PoP) included a control stage without laccase and mediator. A sample of O-O-L pulp was treated with 1.5% NaOH (pulp dry weight) for 1 h at 60ºC and, after alkalilignin precipitation, low molecular mass compounds were extracted (methyl *tert*-butyl ether, pH 5) and analyzed by GC/MS using a DB-5HT column (15 m x 0.25 mm) programmed 50-100ºC (30ºC/min) and 100-300ºC (5ºC/min). Residual lignins were isolated after each stage of the two sequences (and from the alkali-treated pulp) by cellulase hydrolysis, and purified with protease, dimethylacetamide (DMAC), and 0.5 M NaOH (Ibarra et al., 2004). In addition to pulp lignins, MWL was isolated from eucalypt wood, and kraft lignin was recovered from the cooking liquor.

FTIR spectra were obtained in a Bruker IF-28 spectrophotometer using 1 mg of lignin, and baseline was subtracted between valleys *ca* 1850 and 900 cm^{-1}. Pyrolyses were performed at 550ºC (10 s) using a CDS Pyroprobe coupled to Agilent GC and MS equipments. A DV-1701 column (60 m x 0.25 mm), programmed from 45ºC (4 min) to 280ºC at 4ºC/min and 15 min at 280ºC, was used. NMR spectra were recorded at 25ºC in a Bruker AVANCE 500 MHz equipped with a z-gradient triple probe. Fourty mg of lignin were dissolved in 0.75 ml of DMSO-*d6*, and ^{1}H-NMR and 2D-NMR spectra were recorded including HSQC, HSQC-TOCSY and HMBC experiments. Signals were assigned by combining the results of the different spectra and comparison with the literature (Capanema et al., 2001; Liitiä et al., 2003; Ralph et al., 2004).

III. RESULTS AND DISCUSSION

Residual lignins were enzymatically-isolated after the different stages of the enzyme-containing and control sequences (yielding crude, O-O, O-O-L, O-O-L-Q-PoP, O-O-a, and O-O-a-Q-PoP pulps) purified, and analyzed by FTIR, Py-GC/MS, ^{1}H-NMR and 2D-NMR, together with eucalypt MWL and kraft lignin. The structure of lignin, as revealed by the ratio between β-O-4, syringaresinol and phenylcoumaran substructures identified by HSQC NMR, was similar in crude pulp (79:19:2) and wood (80:18:2), although some increase in syringyl-to-guaiacyl (S/G) ratio was revealed by Py-GC/MS and NMR (S/G over 3 in pulp). By contrast, a strong modification of lignin in terms of units (higher S/G ratio) and linkages, resulting in a very different ratio between the above substructures (11:89:0), was observed in the kraft lignin. This lignin included up to 85% phenolic hydroxyls, estimated by ^{1}H-NMR of acetylated samples, compared with only 13% in the residual lignin.

Lignins with similar chemical structure were isolated from O-O and O-O-a pulps, confirming that the control stage did not affected lignin. The lignin from oxygen-delignified pulp showed FTIR spectra and Py-GC/MS profiles (**Fig. 1 A** and **B**) similar to those of lignin from crude pulp. The FTIR spectra revealed higher intensities of S (1329 cm^{-1}) than G bands (1263-1266 cm^{-1}). Py-GC/MS also showed a predominance of compounds derived from S units, such as syringol, 4-vinylsyringol, trans-propenylsyringol and acetosyringone. The HSQC spectra evidenced some changes in lignin due to the action of oxygen, including modification of the ratio between β-O-4, syringaresinol and phenylcoumaran substructures (84:16:0). A complete identification of these side-chain signals (**Fig. 2B**) was posible using HSQC-TOCSY NMR(**Fig. 3A**). This was the case of CH-γ signals in β-O-4 substructures that overlapped in the HSQC spectrum but could be identified from TOCSY. The most important changes after the oxygen stage were observed in the aromatic region (**Fig. 2C**) that showed the presence of CH-2 and CH-6 in oxidized S (δ_C/δ_H 107.3-106.7/7.2-7.3 ppm) and G units (δ_C/δ_H 111.6/7.3 ppm and δ_C/δ_H 118.7/7.5 ppm) together with those of normal S (δ_C/δ_H 104.7/6.7 ppm) and G units (δ_C/δ_H 111.7/7.0 ppm and δ_C/δ_H 119.6/6.8 ppm) found in crude pulp lignin and MWL. Moreover, new signals of muconate-type structures at δ_C/δ_H 128.4-129.6/7.2 ppm appeared after the oxygen stage.

The strongest differences between the lignins isolated from eucalypt pulp were observed after the laccase-mediator stage. The whole HSQC spectrum (**Fig. 2D**) showed that, with the same amount of aromatic CH (7-8%), lignin from the enzymatically-treated pulp had much lower oxygenated aliphatic structures than control lignin (**Fig. 2A**). Moreover, this lignin basically consisted of β-O-4 substructures (**Fig. 2E**). The decrease of oxygenated aliphatic signals suggested oxidative degradation of lignin side-chains. Surprisingly, no modification of the amount of phenolic hydroxyls (estimated by acetylation) was found after the laccase-mediator treatment of

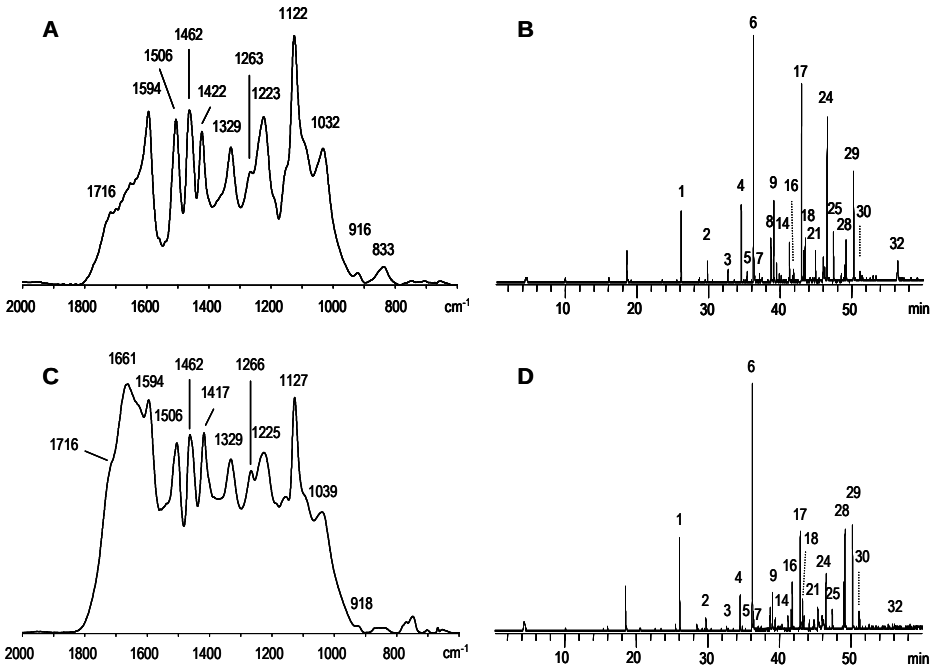

Fig. 1. FTIR spectra (**left**) and Py-GC/MS (**right**) of residual lignin after the laccase-mediator stage (O-O-L pulp, **C** and **D**) and control (O-O-a pulp, **A** and **B**). Py-GC/MS peaks: 1, guaiacol; 2, 4-methylguaiacol; 3, ethylguaiacol; 4, 4-vinylguaiacol; 5, eugenol; 6, syringol; 7, *cis*-isoeugenol; 8, *trans*-isoeugenol; 9, methylsyringol; 14, ethylsyringol; 16, acetoguaiacone; 17, 4-vinylsyringol; 18; guaiacylacetone; 21, *cis*-propenylsyringol; 24, *trans*-propenylsyringol; 25, syringaldehyde; 28, acetosyringone; 29, syrinylacetone; 30, propiosyringone; 32, *trans*-synapaldehyde.

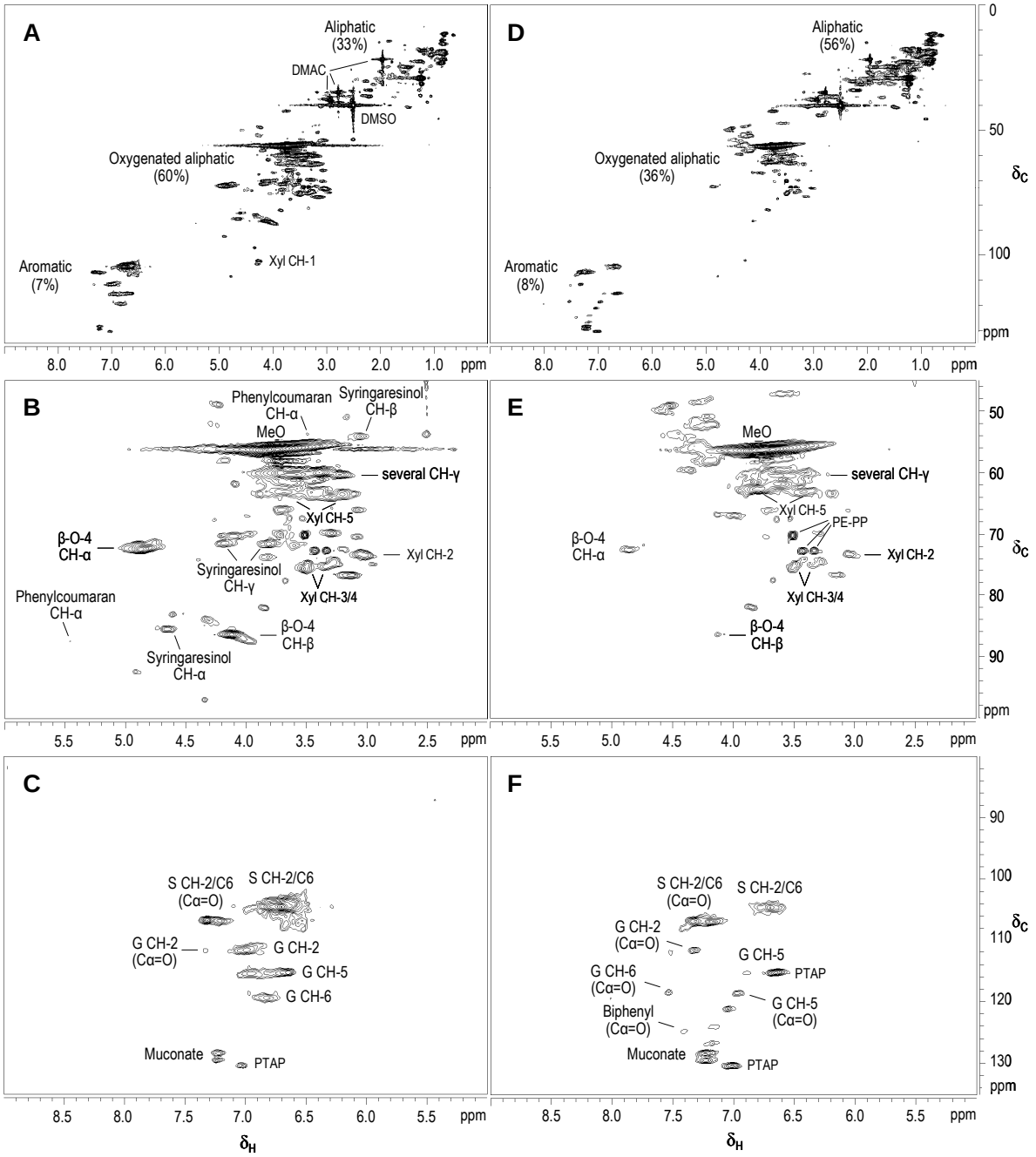

Fig. 2. HSQC spectra of residual lignins after the laccase-mediator stage (O-O-L pulp, **right**) and control (O-O-a pulp, **left**). Whole spectra (**A** and **D**) and expansions of the oxygenated aliphatic (**B** and **E**) and aromatic regions (**C** and **F**). Signals of xylan, DMSO, and three contaminants identified as DMAC, poly(ethylene-propylene) (PE-PP) and *para-tert*-alkylphenol (PTAP), were also found.

pulp. This residual lignin showed a strong conjugated carbonyl band at 1661 cm^{-1} in the FTIR spectrum, and an increase of oxidized products after Py-GC/MS **(Fig. 1C** and **D**) when compared with the control (**Fig. 1A** and **B**). These results agreed with a higher intensity of the HSQC signals assigned to CH-2 and CH-6 in S and G units with conjugated carbonyls (**Fig. 2E**) than found in the control (**Fig. 2B**). In fact, all the G aromatic signals corresponded to units with a conjugated carbonyl (including that of CH-5 at δ_C/δ_H 118.6/7.0 ppm). The above carbonyls mainly corresponded to acetosyringone terminal structures, as found in HMBC NMR experiments showing correlations between the H with δ_H 7.2-7.3 ppm and the ketone C with δ_C 197.9 ppm, whereas this H did not correlate with a side-chain C (as observed at δ_C/δ_H 72.3/6.7 ppm for the non-oxidized S units) (**Fig. 3B** and **C**). Finally, an increase of the two muconate HSQC signals was observed after laccase-mediator treatment (correlation between the muconate olefinic-H and carbonyl-C was found in the HMBC spectra, **Fig. 3B**). This increase indicates oxidative opening of lignin aromatic rings by the laccase-mediator treatment, whereas the strong increase of acetosyringone structures indicate oxidative degradation of lignin side-chains.

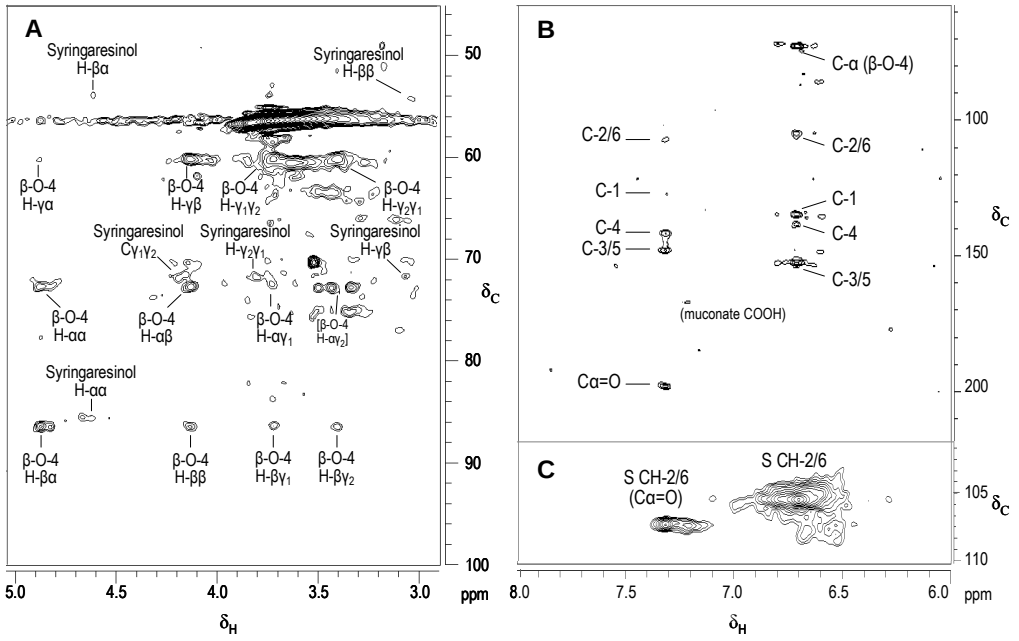

Fig. 3. Details of eucalypt residual lignin HSQC-TOCSY spectrum (δ_C/δ_H 45-100/2.9-5.1 ppm region) showing ^1H-^1H correlations that confirmed identification of side-chain signals **(A),** HMBC spectrum showing multiple-bond ^1H-^{13}C correlations between H-2/6 in oxidized and non-oxidized S units with different C in the δ_C/δ_H 60-220/6-8 ppm region **(B)**, and HSQC spectrum showing the CH-2/6 signals in oxidized (δ_C/δ_H 107.3-106.7/7.2-7.3 ppm) and non oxidized S units (δ_C/δ_H 104.7/6.7 ppm) whose HMBC correlations are shown in **B** (**C**).

The final peroxide stage in the enzyme-containing sequence removed most of the altered lignin, as revealed by FTIR and NMR, and both syringaresinol (10%) and phenylcoumaran (3%) substructures were observed. This was mainly due to the alkaline conditions used, as shown by analyzing the solubilized and residual lignins after an alkaline extraction of the O-O-L pulp. The alkalilignin showed strong FTIR band at 1661 cm^{-1} and HSQC aromatic signals of oxidized S and G units, together with the absence of syringaresinol and phenylcoumaran signals in the HSQC oxygenated aliphatic region. The alkaline extract also included a series of low molecular mass oxidized phenolic compounds (including vanillic and syringic acids) derived from the laccase-mediator attack. On the other hand, the residual lignin obtained after the alkaline treatment was more similar to lignin isolated after the peroxide stage. Although this alkaline treatment lowered the kappa number to 5.4 (a value similar to that found at the end of the O-O-L-Q-PoP sequence, and lower than found after the control sequence) the pulp brightness only attained 73.0% ISO. An additional effect of peroxide was to increase brightness up to 91.2% ISO taking advantage from the alteration of lignin by the laccase-mediator treatment.

IV. ACKNOWLEDGEMENT

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